AD_____

AWARD NUMBER:
W81XWH-06-1-0533

TITLE:
Inflammatory markers and breast cancer risk

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University of Pittsburgh
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REPORT DATE:
July 2010

TYPE OF REPORT: Annual

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2010	Annual	1 JUL 2009 - 30 JUN 2010

4. TITLE AND SUBTITLE
Inflammatory markers and breast cancer risk

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0533

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Brenda Diergaarde, PhD, Eleanor Feingold, PhD, and Robert Ferrell, PhD

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pittsburgh

Pittsburgh, PA 15260

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Material Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Mammographic breast density is one of the strongest known risk factors for breast cancer, and a marker of cancer risk for both breasts. Information on the etiology of breast density is currently limited. To gain further insight into the role of inflammatory cytokines in the etiology of breast density, this study investigates associations between circulating cytokine levels, genetic variation in cytokine genes, and breast density using data and samples from the Mammograms and Masses Study (MAMS). This report provides information on the progress made during the fourth year (no-cost extension) of the grant. We evaluated associations between inflammatory markers interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-alpha), and C-reactive protein (CRP) and mammographic density among 542 postmenopausal MAMS participants. IL-6, TNF-alpha, and CRP levels were not independently associated with dense breast area, nondense breast area or percent density in this study population. The results of this analysis were recently accepted for publication in *Breast Cancer Research and Treatment*.

15. SUBJECT TERMS
Breast cancer; breast density; cytokines; genetic variation

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	19	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION

Mammographic density is one of the strongest known risk factors for breast cancer, and a marker of cancer risk for both breasts [1, 2]. Information on the etiology of breast density is currently limited, and the biological mechanism by which mammographic density is associated with breast cancer risk is unclear. Various evidence suggest that exposure to sex hormones, estrogens in particular, may be an important factor in breast density. Changes in density have been observed in response to hormone replacement therapy use and use of tamoxifen [3, 4]. Pro-inflammatory cytokines, specifically tumor necrosis factor (TNF)-α and interleukin (IL)-6, have emerged as critical regulators of estrogen synthesis in breast tissues [5], and may so affect breast density and breast cancer risk. In line with this, polymorphisms in *IL6* have been found associated with breast cancer risk and to modify the association between estrogen and aspirin and breast cancer risk [6]. To gain further insight into the role of inflammatory cytokines in the etiology of breast density, this study investigates associations between circulating cytokine levels, genetic variation in cytokine genes, and breast density. Existing data and banked specimens from women who participated in a recently completed, cross-sectional study on hormones and breast density, the Mammograms and Masses Study (MAMS), are used.

BODY

- **Circulating levels of inflammatory markers and mammographic density**

We analyzed the cytokine level data and prepared and submitted a manuscript on inflammatory markers and breast density in postmenopausal women to *Breast Cancer Research and Treatment*. The manuscript was recently accepted for publication by this journal (Reeves KW, Weissfeld JL, Modugno F, and Diergaarde B. Circulating levels of inflammatory markers and mammographic density among postmenopausal women. *Breast Cancer Res Treat*. 2010 Nov 11. [Epub ahead of print] PMID: 21069450).

Summary:
Purpose: Mammographic density is strongly associated with breast cancer risk. Inflammation is involved in breast carcinogenesis, perhaps through effects on mammographic density. We evaluated associations between inflammatory markers interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and C-reactive protein (CRP) and mammographic density among postmenopausal women.

Methods: Plasma IL-6, TNF-α, and CRP levels were measured in 145 women with benign breast disease (benign controls) and 397 women with a negative screening mammogram (well controls) enrolled in the Mammograms and Masses Study. Associations between the inflammatory markers and mammographic density were evaluated separately for benign and well controls through correlation analyses and linear regressions.

Results: Age-adjusted mean CRP levels were higher among benign controls (2.07 μg/mL) compared to well controls (1.63 μg/mL; p=0.02), while IL-6 and TNF-α levels were similar between groups. Using linear regression, IL-6, TNF-α, and CRP were not statistically

significantly associated with dense breast area within either group. Statistically significant positive associations were observed between all three markers and nondense breast area in both groups; statistically significant negative associations were observed between IL-6 and percent density among benign controls, and between all three markers and percent density among well controls. These associations were all attenuated and non-significant upon adjustment for body mass index.

Conclusion: IL-6, TNF-α, and CRP levels were not independently associated with dense breast area, nondense breast area or percent density in this study population. Our results suggest that these inflammatory factors do not impact breast carcinogenesis through independent effects on mammographic density.

We excluded premenopausal women from these analyses because a) mammographic density changes during the menstrual cycle [7] and specific information on day of the menstrual cycle at time of mammogram was not available for the study participants; and, b) the number of premenopausal women was relatively small, too small to meaningfully analyze separately.

A copy of the manuscript is appended and contains details on the study population, data collection, the laboratory assays used to measure the levels of IL-6, TNF-α, and CRP, statistical analysis of the data, and the results and conclusions.

- **Variation in inflammation-related genes and mammographic density**

Thus far, MAMS study participants have been genotyped for in total 45 single nucleotide polymorphisms (SNPs) located in or near the following inflammation-related genes: *IL6* (9 SNPs), *IL6R* (12 SNPs), *IL6ST* (7 SNPs), *TNF-α* (1 SNP), *TNFRSF1A* (7 SNPs), and *TNFRSF1B* (9 SNPs). *IL6ST* was included because IL-6 acts by binding to IL-6R which must associate with gp130 (coded for by *IL6ST*) in order for signal transduction to occur.

Putative functional SNPs were selected using public databases [e.g., Genome Variation Server (http://gvs.gs.washington.edu/GVS/) and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/)], and literature search. Additionally, for each gene except *TNF-α*, tagSNPs capturing common variants in the gene region were selected using data from the International HapMap project (www.hapmap.org; CEU population) and Haploview's Tagger [8, 9] [criteria used: minor allele frequency (MAF)>0.05 and pairwise correlation $r^2 \geq 0.80$]. All genotyping was performed at the University of Pittsburgh Genomics and Proteomics Core Laboratories (Pittsburgh, PA). SNP rs1800629 (*TNF-α* -308 G/A), was assessed using TaqMan (Assay ID: C___7514879_10; Applied Biosystems, Foster City, CA). All other SNPs were genotyped using MassARRAY® iPLEX Gold (Sequenom, Inc., San Diego, CA). Sample duplicates (*N*=36) were included to monitor genotyping quality; no discrepant genotypes were observed. Analyses were restricted to women with genotyping call rates of ≥90%. Two study participants were excluded based on <90% call rates.

We are currently analyzing the existing genetic data and plan to evaluate associations between SNPs and mammographic density measures (*i.e.*, dense breast area, nondense area, and percent

density). We will also explore whether these associations are modified by non-steroidal anti-inflammatory drug (NSAID) use or hormone replacement therapy use.

The study population is restricted to MAMS participants with a negative routine screening mammogram (well controls), who had no previous cancer, available mammogram and questionnaire data, and includes only Caucasian postmenopausal women. In total, *N*=372 MAMS participants fulfilled these criteria but two study participants were additionally excluded based on <90% genotyping call rates leaving a total of 370 participants. Selected characteristics of the study population are presented in Table 1 (appended).

Deviation from Hardy-Weinberg equilibrium is assessed with the Chi-square goodness-of-fit test. With the exception of rs2228576 in *TNFRSF1A* (*P*=0.0002) and rs653667 in *TNFRSF1B* (*P*=0.0013), all SNPs were in Hardy-Weinberg equilibrium.

Our preliminary analyses focused on associations with percent density only. Linear regression models were used to examine the relationship between each SNP and percent density. Because the number of rare-allele homozygotes in some cases was relatively small, heterozygotes and rare-allele homozygotes were combined in the analyses (common allele homozygotes were used as the reference group). Percent density was square-root transformed to normalize the distribution. For ease of interpretation, the presented means were transformed back to the original scale. To determine if there was a linear trend with increasing variant alleles, *P* values were also calculated with a linear regression model based on the number of copies of rare alleles (0, 1, 2). All models were adjusted for age (continuous), body mass index (continuous), hormone therapy use (never, past, current), NSAID use (no, yes), pregnancy for at least 6 months (no, yes), and previous biopsy (no, yes). All significance tests were two-sided; *P* values <0.05 were considered statistically significant. All analyses were performed with use of the SAS® statistical software package (SAS version 9.2, SAS Institute Inc., Cary, NC).

Preliminary results (for percent density only): None of the evaluated SNPs in *IL6* and *TNF-α* were significantly associated with percent density in our study population. However, two SNPs located in *IL6R*, rs11265608 and rs64227627, and one in *IL6-ST*, rs11574780, were statistically significantly associated with percent density. For both rs11265608 and rs64227627, mean percent density was significantly higher among women with at least one rare allele than among women homozygous for the common allele (*P*=0.01 and *P*=0.03, respectively). For rs11574780, mean percent density was significantly higher among women homozygous for the common allele (*P*=0.03).

KEY RESEARCH ACCOMPLISHMENTS

- We found that circulating levels of IL-6, TNF-α, and CRP were not independently associated with dense breast area, nondense breast area or percent density among women with benign breast disease or among women with a negative screening mammogram in our study population. We did observe statistically significant, positive associations between these inflammatory factors and nondense breast area and negative associations with percent density in age-adjusted analyses, though further adjustment for body mass index caused these associations to be attenuated and

non-significant among both groups. Adjustment for additional covariates did not affect these estimates further.

- The preliminary results from the genetic data analysis suggest that common variation in *IL6R* and *IL6ST* is associated with percent density in healthy Caucasian postmenopausal women.

REPORTABLE OUTCOMES

Manuscript: Reeves KW, Weissfeld JL, Modugno F, and Diergaarde B. Circulating levels of inflammatory markers and mammographic density among postmenopausal women. *Breast Cancer Res Treat*. 2010 Nov 11. [Epub ahead of print] PMID: 21069450.

Poster/abstract: Brand, H., Weissfeld, J.L., and Diergaarde, B. Common variation in inflammation-related genes and mammographic density in postmenopausal women. Poster presented at the *American Society of Preventive Oncology 34th Annual Meeting*, Bethesda, MD, March 2010.

CONCLUSION

- **Circulating levels of inflammatory markers and mammographic density**

IL-6, TNF-α, and CRP plasma levels were not independently associated with dense breast area, nondense breast area or percent density in our study population, which suggests that these inflammatory factors do not impact breast carcinogenesis through independent effects on mammographic density.

IL-6, TNF-α, and CRP may play an important role in breast carcinogenesis, but it is difficult to separate the effects of the inflammation markers and body mass index/obesity when evaluating their impact on mammographic density. Body mass index is negatively associated with percent density [10-12], and positively associated with nondense breast area [10, 13] and IL-6, TNF-α, and CRP [14-16]; these associations were apparent in our study population as well. Obesity is characterized by the infiltration of macrophages in adipose tissue, and these macrophages are an important source of TNF-α and IL-6 [17, 18]. Smaller quantities of TNF-α and IL-6 are produced by preadipocytes and adipocytes [19].

If body mass index and circulating levels of IL-6, TNF-α, and CRP are not on the same causal pathway, then our adjustment for body mass index is both necessary and appropriate; the conclusion of our results would be that there is truly no independent relationship between these inflammatory markers and nondense breast area and percent density. Alternatively, if body mass index and these inflammatory markers affect nondense breast area and percent density through a shared causal pathway, then adjustment for body mass index would not be appropriate. In this case we would have to conclude that IL-6, TNF-α and CRP are positively associated with nondense breast area and negatively associated with percent density, as indicated in our age-adjusted regressions. Future research will be required to determine whether or not body mass index and these inflammatory markers are on the same causal pathway for mammographic density and/or breast cancer. During our second no-cost extension period we plan to expand the

panel and look at additional markers of inflammation and/or obesity, including IL10, leptin, adiponectin, and MCP-1, to further investigate the relationship between inflammation and breast density.

- **Variation in inflammation-related genes and mammographic density**

  Polymorphisms in *IL6, TNF-α* and the genes that code for their receptors may alter exposure to estrogens and so affect mammographic density. In line with this, our preliminary results suggest that common variation in *IL6R* and *IL6ST* is associated with percent density in healthy Caucasian postmenopausal women. Identification of the genes (and within the genes the functional polymorphisms) that affect breast density will likely provide further insights into the biology of the breast and may identify potential targets for breast cancer (chemo)prevention.

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APPENDICES

- Manuscript: Reeves KW, Weissfeld JL, Modugno F, and Diergaarde B. Circulating levels of inflammatory markers and mammographic density among postmenopausal women. *Breast Cancer Res Treat*. 2010 Nov 11. [Epub ahead of print] PMID: 21069450.

- Table 1

Breast Cancer Res Treat
DOI 10.1007/s10549-010-1249-5

EPIDEMIOLOGY

Circulating levels of inflammatory markers and mammographic density among postmenopausal women

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Received: 14 July 2010 / Accepted: 27 October 2010

Abstract Mammographic density is strongly associated with breast cancer risk. Inflammation is involved in breast carcinogenesis, perhaps through effects on mammographic density. We evaluated associations between inflammatory markers interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and C-reactive protein (CRP) and mammographic density among postmenopausal women. Plasma IL-6, TNF-α, and CRP levels were measured in 145 women with benign breast disease (benign controls) and 397 women with a negative screening mammogram (well controls) enrolled in the Mammograms and Masses Study. Associations between the inflammatory markers and mammographic density were evaluated separately for benign and well controls through correlation analyses and linear regressions. Age-adjusted mean CRP levels were higher among benign controls (2.07 μg/ml) compared to well controls (1.63 μg/ml; $P = 0.02$), while IL-6 and TNF-α levels were similar between groups. Using linear regression, IL-6, TNF-α, and CRP were not statistically significantly associated with dense breast area within either group. Statistically significant positive associations were observed between all three markers and nondense breast area in both groups; statistically significant negative associations were observed between IL-6 and percent density among benign controls, and between all three markers and percent density among well controls. These associations were all attenuated and non-significant upon adjustment for body mass index. IL-6, TNF-α, and CRP levels were not independently associated with dense breast area, nondense breast area, or percent density in this study population. Our results suggest that these inflammatory factors do not impact breast carcinogenesis through independent effects on mammographic density.

Keywords Interleukin-6 (IL-6) · Tumor necrosis factor-α (TNF-α) · C-reactive protein (CRP) · Mammographic density · Postmenopausal

Abbreviations

ANOVA	Analysis of variance
BMI	Body mass index
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
IL-6	Interleukin-6
LCBR	Laboratory for Biochemsitry Research
MAMS	Mammograms and Masses Study
NSAID	Non-steroidal anti-inflammatory drug
TNF-α	Tumor necrosis factor alpha

Introduction

Mammographic density is positively associated with breast cancer risk, and it may represent an associated phenotype

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Published online: 11 November 2010

for this disease [1]. Mammographic density refers to the amount of connective and epithelial tissue present in the breast relative to fat as viewed on a mammogram [2, 3]. Two common measurements of mammographic density are dense breast area and percent density. Percent density is the more frequently used measure, yet dense breast area also is strongly related to breast cancer risk [4–6]. The heritability of percent density is estimated to be 63% [7]. Thus, more than one-third of the variability of breast density is influenced by other, potentially modifiable, factors. Indeed, studies have demonstrated that mammographic density changes in response to factors such as use [8, 9] or cessation [10] of hormone therapy. Mammographic density also changes during the menstrual cycle [11].

Estrogen plays a critical role in breast carcinogenesis, and exposure to both endogenous [12, 13] and exogenous [14, 15] estrogens is positively associated with breast cancer risk. In recent years evidence has emerged that breast cancer etiology may also have an inflammatory component. Inflammatory factors might influence breast cancer risk through their effects on the estrogen pathway. For example, breast cancer risk is approximately 20% lower among women who regularly use non-steroidal anti-inflammatory drugs (NSAIDs) [16–19]. Aspirin use decreases risk of progression to breast cancer among women with benign breast disease [20]. NSAIDs block cyclooxygenase-2 (COX-2), an enzyme that converts arachidonic acid into prostaglandins, which in turn trigger increased estrogen formation in adipose tissue [21]. The inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) increase the production of aromatase, the enzyme responsible for estrogen production in adipose tissue via conversion of androstenedione to estrone [22, 23]. This action of IL-6 and TNF-α is especially important in postmenopausal women, as estrone is the primary form of estrogen produced after the menopause [24]. Levels of the acute-phase inflammatory marker C-reactive protein (CRP) are decreased when COX-2 action is inhibited [25]. Thus, IL-6, TNF-α, and CRP may provide a link between the inflammatory and estrogen pathways thought to be important to the development of breast cancer.

Studies examining circulating levels of IL-6, TNF-α, or CRP in relation to breast cancer risk have provided inconsistent results. Some studies report no association between IL-6 and risk of breast cancer [26, 27] or cytologic atypia [28], while another observed elevated IL-6 levels among breast cancer cases with insulin resistance [29]. Five studies reported no significant association between TNF-α and breast cancer [26, 29–32], while one found decreased production of TNF-α from T lymphocytes in breast cancer patients [33] and another observed increased levels of TNF-α among breast cancer cases [34]. Most studies of CRP and breast cancer risk found no statistically significant association [26, 27, 35–37], though one reported a significant positive association [38]. An additional study reported a weak, positive association between CRP in nipple aspirate fluid and Gail score, an indicator of breast cancer risk [39]. Many of the published studies on IL-6, TNF-α, and CRP and breast cancer risk, however, are affected by numerous biases and limitations, including small sample sizes, inadequate description of laboratory procedures and quality control, and failure to control for potential confounders. Additionally, the relationship between these inflammatory factors and benign breast disease has not been described. Inflammation is an extremely complex process, and the exact mechanisms by which inflammation relates to the occurrence of breast cancer are not yet known. It may be that inflammatory factors impact breast carcinogenesis through a pathway which includes mammographic density, yet this has not been explored in published reports.

We evaluated associations between IL-6, TNF-α, and CRP levels and measures of mammographic density (dense breast area, nondense breast area, and percent density) in a large sample of postmenopausal women on whom extensive covariate data were available. To our knowledge, no previous studies have investigated associations between IL-6, TNF-α or CRP, and mammographic density.

Materials and methods

Study population

We conducted a cross-sectional investigation using controls participating in the Mammograms and Masses Study (MAMS), a case–control study on hormones and mammographic density. Details of MAMS have been described elsewhere [40, 41]. Briefly, women were eligible for MAMS if they were 18 years or older and were visiting Magee-Womens Hospital (Pittsburgh, PA) or a Magee Womancare Center in the greater Pittsburgh area for one of the following: (a) a breast biopsy, (b) an initial surgical consultation after breast cancer diagnosis, or (c) a routine screening mammogram. Women were excluded if they reported a prior cancer history other than non-melanoma skin cancer, drank more than five alcoholic beverages per day, or weighed less than 110 lb or more than 300 lb. Recruitment took place from September 2001 to May 2005. Pathology reports were used to determine disease status (benign breast disease, in situ breast cancer, invasive breast cancer) for those undergoing a breast biopsy and/or surgery. The MAMS study population consists in total of 1,133 women: 264 women with in situ or invasive breast cancer (cases), 313 women with benign breast disease (benign controls), and 556 women with a negative screening

mammogram (well controls). The University of Pittsburgh Institutional Review Board reviewed and approved the study protocol, and all study participants provided written informed consent.

Both benign and well controls were included in the present study ($N_{total} = 869$). For the current analyses, we subsequently excluded all women who were not postmenopausal ($N = 222$), had no available mammogram data ($N = 53$), did not complete the questionnaire ($N = 26$), reported a prior history of cancer after enrollment into MAMS ($N = 9$), had no available plasma sample ($N = 8$) or whose blood draw was more than 180 days from their mammogram date ($N = 9$), leaving a final total of 145 benign controls and 397 well controls. We excluded premenopausal women because fluctuating hormone levels during the menstrual cycle can affect cytokine levels in premenopausal women and specific information on day of the menstrual cycle at time of mammogram was not available.

Data collection

Information on medical history, reproductive history, lifestyle factors such as smoking status and alcohol intake, demographic characteristics, medication use, and family history of breast cancer was collected using a self-administered questionnaire. Women were assumed to be postmenopausal if they had no periods in the year before enrollment, had ever used hormone therapy, had had a bilateral oophorectomy, or were 60 years or older at enrollment. Women who reported a hysterectomy without bilateral oophorectomy were considered to be postmenopausal if they had ever used hormone therapy or were 50 years or older at hysterectomy. Age at menopause was set to age at which menstrual periods ended, age at a bilateral oophorectomy, or age of first use of hormone therapy, whichever came first. For women who had a hysterectomy without bilateral oophorectomy, age at menopause was set to age at which they first used hormone therapy or first had menopausal symptoms, whichever came first. If neither occurred and age at hysterectomy was 50 years or older, then age at menopause was age at hysterectomy. Height and weight were measured by a research nurse using a stadiometer and a standard balance beam scale while participants wore light clothing and no shoes. Body mass index (BMI) was computed as weight (in kg) divided by height squared (in meters). The summary variable 'current NSAID use' was created as described previously [41]. A non-fasting, 40 ml sample of peripheral blood was collected from the study participants at enrollment. All samples were processed immediately at the Magee-Womens Hospital Clinical Research Center and stored at $<-70°C$. Blood samples were taken an average of 34 days (SD 29 days) after the mammogram. The majority (58.5%) of blood samples was collected within 31 days of the mammogram; 94% were collected within 90 days of the mammogram. The time interval from mammogram to blood collection did not differ significantly between benign and well controls ($P = 0.47$).

Laboratory assays

Circulating levels of IL-6, TNF-α, and CRP were measured in frozen stored EDTA plasma samples by the Laboratory for Clinical Biochemistry Research (LCBR) at the University of Vermont (Colchester, VT). Samples were shipped to the LCBR packed in dry ice using overnight courier service. Investigators at the LCBR were blinded to the identity, demographic and risk factor characteristics, and mammographic density status of the samples. To evaluate assay reproducibility, 36 masked, duplicate samples (6.6% of total study samples) were randomly distributed throughout the batch of samples. Plasma IL-6 levels were measured using a high sensitivity enzyme-linked immunosorbent assay (ELISA; Human IL-6 Quantikine® HS, HS600B) from R&D Systems (Minneapolis, MN). The detectable limit for IL-6 was 0.10 pg/ml, and the average coefficient of variation (CV) was 16.0%. TNF-α levels were measured by a singleplex immunoassay using Luminex technology (Human Cytokine LINCOplex Kit Singleplex TNF-α, HCYTO-60K-1TNFA; Linco Research, Inc., St. Charles, MO). This assay can measure TNF-α concentrations $\leq$3.2 pg/ml, and the average CV was 10.8%. CRP levels were measured using the BNII nephelometer from Dade Behring utilizing a particle-enhanced immunonepholometric assay. This assay has a detection limit of 0.16 μg/ml, and the average CV was 9.6%.

Mammographic density measurements

Copies of participants' most recent screening mammograms were obtained with their permission. The assessment of mammographic measures has been described in detail elsewhere [40]. Briefly, one expert reader read all mammograms, which were copies of the original films. This reader was masked to the identity, status (benign control, well control), and demographic and risk factor characteristics of the subject. Total breast area and all dense regions were measured using a compensating polar planimeter (LASICO) on the craniocaudal view with the side of breast (right or left) randomly chosen for each participant. Dense breast area is the sum of all dense regions; nondense breast area was calculated by subtracting dense breast area from total breast area; percent density was calculated by dividing dense breast area by total breast area and multiplying that by 100. A subjective measure of film quality was also reported (excellent, good, fair, poor, very poor, extremely poor) by the expert reader. In a separate reproducibility

study using mammograms from 28 MAMS participants, intraclass correlation coefficients for dense breast area, total breast area and percent density were $\rho = 0.86$, $\rho = 0.99$, and $\rho = 0.89$, respectively [40].

Statistical analysis

All analyses were performed separately for benign controls and well controls. Differences between benign and well controls were assessed using chi-square tests for categorical variables and *t*-tests or analysis of variance (ANOVA) for continuous variables. The normality of the distribution of circulating IL-6, TNF-α, and CRP levels, and dense breast area, nondense breast area, and percent density was assessed graphically using quantile–quantile plots. To improve normality, natural log transformations were applied to the inflammatory markers and square root transformations were applied to the mammographic density measures. Pearson's correlation coefficient was used to examine the correlation between cytokine levels and mammographic measures; Fisher's z transformations were used to test differences between the correlation coefficients. Linear regression was used to further assess the association between each inflammatory marker and mammographic density. The assumptions needed for linear regression were met. Unadjusted, age-adjusted, age- and BMI-adjusted, and multivariable-adjusted regression models were run for each combination of inflammatory marker and mammographic density measure. The multivariable model included covariates found to be associated with mammographic density and/or breast cancer in previous studies: age (continuous), BMI (<25 kg/m^2, 25 to <30 kg/m^2, ≥ 30 kg/m^2), race (white, other), smoking (never, former, current), current NSAID use (nonuser, user), first-degree relative with breast cancer (no, yes), age at menarche (≤ 12, >12), age at menopause (<50, ≥ 50), type of menopause (natural, hysterectomy without oophorectomy, hysterectomy with uni- or bilateral oophorectomy), prior breast biopsy (no, yes), ever been pregnant (no, yes), and postmenopausal hormone therapy use status (never, former, current). We subsequently repeated the regressions stratified by BMI, current NSAID use, and time between blood draw and mammogram, and separately among participants with high quality mammograms. *P* values <0.05 were considered statistically significant. Analyses were performed using Stata (version 10.0; Stata Corporation, College Station, TX) and SAS (version 9.2; SAS Institute Inc., Cary, NC) software.

Results

The study population consisted of two groups: 145 women with benign breast disease (benign controls) and 397 women with a negative screening mammogram (well controls). Characteristics of the study population by control status are presented in Table 1. Mean age among benign controls was statistically significantly lower than among well controls. Additionally, benign controls reported having had a prior breast biopsy, to be older than 12 years of age at menarche, to have gone through menopause before age 50, and to be current users of postmenopausal hormone therapy statistically significantly more often than well controls. Current use of NSAIDs was significantly more common among the well controls.

The distributions of the inflammatory markers and the mammographic density measures by control status are shown in Table 2. The age-adjusted geometric mean of CRP was statistically significantly higher among benign controls (2.07 μg/ml) than among well controls (1.63 μg/ml; $P = 0.02$). No significant differences between benign controls and well controls were observed for IL-6 and TNF-α. Regarding the mammographic density measures, age-adjusted mean dense breast area (42.8 vs. 36.1 cm^2; $P = 0.02$) and age-adjusted mean percent density (31.2 vs. 26.0%; $P = 0.01$) were both statistically significantly higher among benign controls than among well controls. Age-adjusted mean nondense breast area did not differ significantly between the two groups (97.8 vs. 108.1 cm^2; $P = 0.13$).

No significant correlations were observed between circulating IL-6, TNF-α, and CRP levels and dense breast area among benign or well controls (Table 3). However, all three cytokines were statistically significantly, positively correlated with nondense breast area in both control groups (benign controls: IL-6: $\rho = 0.32$, $P < 0.001$; TNF-α: $\rho = 0.22$, $P < 0.001$; CRP: $\rho = 0.25$, $P = 0.003$; well controls: IL-6: $\rho = 0.30$, $P < 0.001$; TNF-α: $\rho = 0.26$, $P < 0.001$; CRP: $\rho = 0.36$, $P < 0.001$), and statistically significantly, negatively correlated with percent density among well controls (IL-6: $\rho = -0.20$, $P < 0.001$; TNF-α: $\rho = -0.18$, $P < 0.001$; CRP: $\rho = -0.23$, $P < 0.001$). Among benign controls the correlation with percent density was statistically significant for IL-6 ($\rho = -0.21$, $P = 0.01$) and borderline significant for CRP ($\rho = -0.16$, $P = 0.05$). TNF-α levels were also negatively correlated with percent density among benign controls, yet this association was not statistically significant. No statistically significant differences in correlation coefficients for IL-6, TNF-α, and CRP were observed between benign controls and well controls (Table 3).

Results from the age-adjusted, age- and BMI-adjusted, and multivariable-adjusted linear regression models are presented in Table 4. For both benign controls and well controls, no statistically significant associations were observed between levels of IL-6, TNF-α, and CRP and dense breast area in any of the models but all three

Table 1 Selected characteristics of the study population by control status ($N_{total} = 542$)

	Benign controls ($N = 145$)[a] *N* (%)	Well controls ($N = 397$)[a] *N* (%)	P[b]
Age (years; mean ± SD)	58.3 ± 7.4	62.0 ± 8.1	<0.001
Age (years)			<0.001
Younger than 50	12 (8.3)	4 (1.0)	
50–59	68 (46.9)	180 (45.3)	
60–69	55 (37.9)	135 (34.0)	
70 or older	10 (6.9)	78 (19.7)	
Race: White	136 (93.8)	373 (94.0)	0.94
Body mass index (kg/m^2; mean ± SD)	27.9 ± 5.9	28.3 ± 6.0	0.57
Body mass index (kg/m^2)			0.46
Normal (less than 25.0)	44 (30.6)	131 (33.0)	
Overweight (25.0 to <30.0)	58 (40.3)	137 (34.5)	
Obese (30.0 or more)	42 (29.2)	129 (32.5)	
Smoking status			0.07
Never	81 (53.6)	227 (57.2)	
Former	45 (31.3)	144 (36.3)	
Current	18 (12.5)	26 (6.6)	
Prior breast biopsy	60 (41.7)	57 (14.4)	<0.001
First-degree relative with breast cancer	18 (12.5)	56 (14.2)	0.61
Age at menarche (years)			0.04
12 or younger	58 (40.0)	197 (49.8)	
Older than 12	87 (60.0)	199 (50.3)	
Ever been pregnant	121 (83.5)	332 (83.6)	0.96
Age at first pregnancy lasting ≥6 months			0.36
Never pregnant/no pregnancies ≥6 months	32 (22.2)	80 (20.2)	
Younger than 20	18 (12.5)	35 (8.8)	
20–24	52 (36.1)	143 (36.0)	
25–29	27 (18.8)	89 (22.4)	
30 or older	15 (10.4)	50 (12.6)	
Age at menopause (years)			<0.001
Younger than 50	85 (59.9)	164 (42.2)	
50 or older	57 (40.1)	225 (57.8)	
Type of menopause			0.13
Natural menopause	90 (65.7)	275 (72.4)	
Hysterectomy without oophorectomy	16 (11.7)	48 (12.6)	
Hysterectomy with uni- or bilateral oophorectomy	31 (22.6)	57 (15.0)	
Postmenopausal hormone therapy use			<0.001
Never	27 (18.8)	140 (35.3)	
Former	43 (29.9)	203 (51.1)	
Current (within previous 3 months)	74 (51.4)	54 (13.6)	
Current NSAID use	42 (34.4)	194 (49.2)	0.004

[a] The numbers do not always add up to the total number of benign and well controls due to missing information

[b] Chi-square tests for categorical variables and *t*-tests for continuous variables

inflammatory markers were statistically significantly associated with nondense breast area in the age-only adjusted model (benign controls: IL-6: $\beta = 1.44, P < 0.001$; TNF-α: $\beta = 1.46$, $P = 0.007$; CRP: $\beta = 0.70$, $P = 0.003$; well controls: IL-6: $\beta = 1.49$, $P < 0.001$; TNF-α: $\beta = 1.78$, $P < 0.001$; CRP: $\beta = 1.18$, $P < 0.001$). The associations with nondense breast area became non-significant after additional adjustment for BMI and other variables. Among benign controls, IL-6 was statistically significantly associated with percent density in the age-only adjusted model ($\beta = -0.55$, $P = 0.02$). This association became non-significant upon further adjustment for BMI, and remained non-significant upon adjustment for additional covariates. No significant associations were observed for TNF-α and

Table 2 Distribution of inflammatory markers and mammographic density measures by control status

	Benign controls				Well controls				P^b
	N	Mean (SD)	Median	Age-adjusted transformed mean[a]	*N*	Mean (SD)	Median	Age-adjusted transformed mean[a]	
Inflammatory markers[c]									
IL-6 (pg/ml)	145	2.67 (2.72)	1.97	2.12	397	2.89 (2.91)	1.98	2.17	0.71
TNF-α (pg/ml)	145	3.00 (1.60)	2.59	2.67	394	2.99 (1.83)	2.68	2.63	0.70
CRP (μg/ml)	142	4.16 (8.57)	2.17	2.07	381	2.92 (4.17)	1.47	1.63	0.02
Mammographic density measures									
Dense breast area (cm^2)	145	48.0 (30.6)	44.6	42.8	397	40.9 (26.6)	36.7	36.1	0.02
Nondense breast area (cm^2)	145	106.0 (71.9)	90.5	97.8	397	120.7 (76.3)	100.1	108.1	0.13
Percent density (%)	145	35.2 (18.8)	34.2	31.2	397	29.6 (19.4)	27.5	26.0	0.01

[a] Transformed mean is geometric mean for the inflammatory markers. For the mammographic density variables, the transformed mean is a mean calculated on the square root scale that was subsequently transformed back to the original scale

[b] *P* values from ANOVA, comparing distributions among benign controls to well controls using natural log transformations of the inflammatory markers and square root transformations of the mammographic density variables with adjustment for age

[c] TNF-α levels could not be measured for three well controls; CRP levels could not be measured for three benign controls and 16 well controls

Table 3 Correlations between inflammatory markers and mammographic density measures by control status[a]

	Benign controls			Well controls			P^b
	N	ρ	*P*	*N*	ρ	*P*	
Dense breast area							
IL-6	145	−0.03	0.72	397	−0.06	0.24	0.77
TNF-α	145	0.04	0.64	394	−0.01	0.78	0.59
CRP	142	−0.01	0.95	381	−0.03	0.60	0.83
Nondense breast area							
IL-6	145	0.32	<0.001	397	0.30	<0.001	0.90
TNF-α	145	0.22	<0.001	394	0.26	<0.001	0.65
CRP	142	0.25	0.003	381	0.36	<0.001	0.22
Percent density							
IL-6	145	−0.21	0.01	397	−0.20	<0.001	0.90
TNF-α	145	−0.11	0.19	394	−0.18	<0.001	0.44
CRP	142	−0.16	0.05	381	−0.23	<0.001	0.50

[a] Calculated using Pearson's correlation coefficient with natural log transformations of the inflammatory markers and square root transformations of the mammographic density variables

[b] *P* values for comparison of correlation coefficients between benign controls and well controls

CRP levels with percent density among women with benign breast disease in any model. Among well controls, all three inflammatory markers were statistically significantly associated with percent density in the age-only adjusted model (IL-6: $\beta = -0.54$, $P < 0.001$; TNF-α: $\beta = -0.71$, $P < 0.001$; CRP: $\beta = -0.43$, $P < 0.001$). These associations became non-significant after additional adjustment for BMI and other variables.

Subsequently, regressions were repeated stratified by BMI, current NSAID use, and time between blood draw and mammogram; results were generally similar to those observed overall (data not shown). Additionally, results were similar to those observed in the total populations of benign controls and well controls when regressions were restricted to women with mammograms of good or excellent film quality (data not shown).

Discussion

Plasma levels of IL-6, TNF-α, and CRP were not independently associated with dense breast area, nondense breast area, or percent density among women with benign breast disease or among women with a negative screening mammogram in our study population. We did observe statistically significant, positive associations between these inflammatory factors and nondense breast area and negative associations with percent density in age-adjusted analyses, though further adjustment for BMI caused these associations to be attenuated and non-significant among both groups. Adjustment for additional covariates did not affect these estimates further.

It is of interest that CRP levels were statistically significantly elevated among women with benign breast disease compared to the well controls. This may reflect true effects of benign breast disease on inflammation, or vice versa, or it may relate to the lower NSAID use observed among benign versus well controls. Benign controls were recruited and gave a blood sample at the time of their breast biopsy, and their less frequent NSAID use may reflect instructions given by their physician to avoid NSAID use prior to the biopsy procedure. Dense breast area and percent density were significantly greater among

Table 4 Results of linear regressions of mammographic density measures on inflammatory markers by control status[a]

	Age-adjusted			Age- and BMI-adjusted			Fully adjusted[b]		
	N	β (SE)	*P*	*N*	β (SE)	*P*	*N*	β (SE)	*P*
Benign controls									
Dense breast area									
IL-6	145	−0.13 (0.28)	0.64	144	−0.07 (0.31)	0.83	111	−0.07 (0.39)	0.85
TNF-α	145	0.18 (0.37)	0.63	144	0.26 (0.39)	0.50	111	0.40 (0.50)	0.43
CRP	142	−0.01 (0.16)	0.95	141	0.001 (0.17)	0.99	109	0.16 (0.21)	0.45
Nondense breast area									
IL-6	145	1.44 (0.39)	<0.001	144	0.23 (0.35)	0.52	111	0.09 (0.45)	0.84
TNF-α	145	1.46 (0.53)	0.007	144	0.50 (0.44)	0.26	111	0.03 (0.58)	0.96
CRP	142	0.70 (0.23)	0.003	141	0.16 (0.19)	0.42	109	0.06 (0.24)	0.82
Percent density									
IL-6	145	−0.55 (0.23)	0.02	144	−0.08 (0.23)	0.74	111	−0.03 (0.30)	0.93
TNF-α	145	−0.42 (0.31)	0.18	144	−0.03 (0.29)	0.92	111	0.19 (0.35)	0.62
CRP	142	−0.26 (0.13)	0.06	141	−0.05 (0.13)	0.69	109	0.06 (0.17)	0.71
Well controls									
Dense breast area									
IL-6	397	−0.16 (0.16)	0.32	397	−0.07 (0.17)	0.69	368	−0.15 (0.18)	0.41
TNF-α	394	−0.04 (0.23)	0.87	394	0.09 (0.24)	0.71	365	0.04 (0.25)	0.87
CRP	381	−0.06 (0.11)	0.57	381	0.01 (0.12)	0.96	353	0.02 (0.13)	0.85
Nondense breast area									
IL-6	397	1.49 (0.24)	<0.001	397	0.39 (0.20)	0.05	368	0.34 (0.22)	0.12
TNF-α	394	1.78 (0.34)	<0.001	394	0.35 (0.28)	0.21	365	0.32 (0.30)	0.29
CRP	381	1.18 (0.16)	<0.001	381	0.27 (0.14)	0.06	353	0.29 (0.16)	0.06
Percent density									
IL-6	397	−0.54 (0.14)	<0.001	397	−0.11 (0.14)	0.43	368	−0.13 (0.15)	0.38
TNF-α	394	−0.71 (0.20)	<0.001	394	−0.16 (0.19)	0.39	365	−0.17 (0.20)	0.41
CRP	381	−0.43 (0.09)	<0.001	381	−0.08 (0.10)	0.44	353	−0.07 (0.10)	0.48

[a] Regressions performed using natural log transformations of the inflammatory markers and square root transformations of the mammographic measures

[b] Adjusted for age, race, BMI, smoking, current NSAID use, first-degree relative with breast cancer, age at menarche, age at menopause, type of menopause, prior breast biopsy, ever been pregnant, and postmenopausal hormone therapy use

benign versus well controls. This finding is in agreement with a prior study documenting strong correlation between dense breast area and percent density and history of atypical hyperplasia or lobular carcinoma in situ [42]. These differences support our decision to consider women with benign breast disease separate from women with negative screening mammograms in our analyses.

Our results indicate that IL-6, TNF-α, and CRP do not independently affect breast cancer risk through a pathway that includes mammographic density. The positive and negative age-adjusted associations that we observed with nondense breast area and percent density, respectively, were attenuated and became non-significant when adjusted for BMI. BMI is negatively associated with percent density [43–45], and BMI is positively associated with nondense area [46] and IL-6, TNF-α, and CRP [26, 47, 48]; these associations were apparent in our study population as well (data not shown). Obesity is characterized by infiltration of macrophages in adipose tissue, and these macrophages are an important source of TNF-α and IL-6 [49, 50]. Smaller quantities of TNF-α and IL-6 are produced by preadipocytes and adipocytes [51]. IL-6, TNF-α, and CRP may play an important role in breast carcinogenesis, but it is difficult to separate the effects of the inflammation markers and BMI when evaluating their influences on percent density.

If BMI and circulating levels of IL-6, TNF-α, and CRP are not on the same causal pathway, then our adjustment for BMI is both necessary and appropriate; the conclusion of our results would be that there is truly no independent relationship between these inflammatory markers and nondense breast area and percent density. Alternatively, if BMI and these inflammatory markers affect percent density

through a shared causal pathway, then adjustment for BMI would not be appropriate. In this case we would have to conclude that IL-6, TNF-α, and CRP are positively associated with nondense breast area and negatively associated with percent density, as indicated in our age-adjusted regressions. Future research will be required to determine whether or not BMI and these inflammatory markers are on the same causal pathway for mammographic density and/or breast cancer.

An additional possibility is that percent density is not an appropriate measure for studying etiologic associations between biomarkers and mammographic density. Percent density represents both the number of cells at risk for breast cancer (dense breast area) and the amount of fat tissue (nondense area) in the breast, which is highly correlated with BMI [43]. Therefore, observed associations between percent density and exposures that are strongly associated with BMI may not indicate direct effects of such exposures on the dense breast tissue [43]. The issues related to BMI and percent density do not appear to be unique to IL-6, TNF-α, and CRP, but rather occur with other BMI-associated exposures as well. As a result, it may be more appropriate to use dense breast area as the preferred measure of mammographic density in etiologic studies [43, 45]. In our study population none of the inflammatory factors investigated were related to dense breast area even in unadjusted analyses.

Previous studies provide inconsistent evidence for IL-6, TNF-α, and CRP in relation to breast carcinogenesis. The majority of studies have found no association, yet some found positive associations with IL-6 [29], TNF-α [34], and CRP [38]. Two of these studies [29, 34] did not adjust for BMI, however, which, as discussed above, may or may not confound the observed associations. Based on our results, it is not clear that IL-6, TNF-α, or CRP is independently associated with mammographic density, an associated phenotype for breast cancer.

Limitations of this study primarily relate to the measurement of the inflammatory factors. IL-6, TNF-α, and CRP were all measured at a single time-point, and therefore may not be representative of a participant's usual levels. Circulating levels of IL-6, TNF-α, and CRP could reflect recent changes in general health or medication use. In particular, current use of NSAIDs could greatly impact circulating levels of these factors. However, we were able to control for current use of NSAIDs in our analysis, and recent studies have demonstrated reasonable within-subject stability of serum IL-6, TNF-α, and CRP levels over 1 year [52] and over repeated monthly measurements [53]. Additionally, the correlation between circulating and breast tissue levels of these factors has not been established. Tissue levels might be more relevant to breast carcinogenesis and might possibly show a different association with mammographic density. Finally, external validity is limited by the racial homogeneity, high socioeconomic status, and overall good health of the study population. Our study is strengthened by our large sample size, use of healthy subjects, and the high reliability of our IL-6, TNF-α, CRP, and mammographic density measurements.

Though inflammatory pathways may be important to breast carcinogenesis, our results suggest that the inflammatory markers IL-6, TNF-α, and CRP do not impact breast carcinogenesis through independent effects on mammographic density. Future research is needed to elucidate the exact mechanisms by which inflammation is related to breast cancer risk.

Acknowledgments This work was supported by Department of Defense Grant BC050518 and National Institutes of Health Grant R25 CA057703. Additional support was provided by funds received from the National Institutes of Health/National Center for Research Resources/General Clinical Research Center Grant MO1-RR000056.

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Table 1: Selected characteristics of the study population (N_{total}=370)

	Well controls N(%)
Age (years; mean ± SD)	62. 1 ± 8.2
Age (years)	
Younger than 50	3 (0.8)
50-59	165 (44.6)
60-69	128 (34.6)
70 or older	74 (20.0)
Body mass index (kg/m^2; mean ± SD)	28.1 ± 5.9
Body mass index (kg/m^2)	
Normal (less than 25.0)	124 (33.5)
Overweight (25.0-<30.0)	131 (35.4)
Obese (30.0 or more)	115 (31.1)
Smoking status	
Never	212 (57.3)
Former	138 (37.3)
Current	20 (5.4)
Prior breast biopsy	55 (14.9)
First-degree relative with breast cancer	56 (15.1)
Ever been pregnant	309 (83.5)
Age at menopause 50 years or older[a]	216 (59.5)
Postmenopausal hormone therapy use	
Never	133 (36.0)
Former	188 50.8)
Current (within previous 3 months)	49 (13.2)
Current NSAID use	183 (49.5)
Mammographic density measures	
Dense breast area (cm^2; mean ± SD)	41.2 (26.8)
Nondense breast area (cm^2; mean ± SD)	118.2 (75.2)
Percent density (%; mean ± SD)	30.2 (19.5)

[a] Age at menopause was not available for 7 of the study participants.